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On the role of corticosterone in behavioral disorders, microbiota composition alteration and neuroimmune response in adult male mice subjected to maternal separation stress

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ABSTRACT

Experiencing psychosocial adversities in early life such as maternal separation (MS) increases the risk of psychiatric disorders. Immune-inflammatory responses have imperative roles in the pathophysiology of psychiatric disorders. MS relatively changes the composition of intestinal microbiota leading to an overactivation of the hypothalamic–pituitary–adrenal (HPA) axis, and subsequently increases the corticosterone level. In this study, we aimed to evaluate the role of corticosterone in behavioral changes and microbiota modifications in a mouse model of MS afflicted neuroinflammatory response in the hippocampus. For this purpose, 180 min of MS stress was applied to mice at postnatal day (PND) 2–14 followed by behavioral tests including forced swimming test (FST), splash test, open field test (OFT) and elevated plus maze (EPM) at PND 50–52. For evaluating the role of corticosterone, mice were subjected to adrenalectomy. Using real-time RT-PCR, the expression of inflammatory genes was determined in the hippocampus and colon tissues. We found that MS provoked depressive- and anxiety-like behaviors in adult male mice. In addition, MS was able to active a neuroimmune response in the hippocampus, motivate inflammation and histopathologic changes in the colon tissue and modify the composition of gut microbiota as well. Interestingly, our findings showed that adrenalectomy (decline in the corticosterone level), could modulate the above-mentioned negative effects of MS. In conclusion, our results demonstrated that overactivation of HPA axis and the subsequent increased level of corticosterone could act, possibly, as the deleterious effects of MS on behavior, microbiota composition changes and activation of neuroimmune response.

1. Introduction

Social ties, principally mother-infant relationship during the infantile period, have a critical role in the development of the brain and behavior in adulthood [1]. It is shown that neonate exposure into psychosocial adversities, like maternal separation (MS) stress, dramatically interrupt brain development and augments the risk of neuropsychiatric diseases including anxiety and depression [2].

Activation of hypothalamic–pituitary–adrenal (HPA) axis followed by stress adapts body function has the potential to modulate physiological responses to upcoming tensions through over-release of glucocorticoids [3]. Previous studies have demonstrated that experiencing adversities in early life contributed to overactivation of the HPA axis, and consequently increased corticosterone levels resulting in psychological disorders in later life [3]. In other words, experiencing early life stress like MS leads to persistent and life-long changes in the HPA axis which means that the stress center (HPA axes) sets in a higher point [4,5].

The gut contains a diverse microbial biota, which are mainly anaerobes bacteria, in addition to fungi and viruses [6]. Microflora has

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an approved central role in the regulation of emotion and behaviors [7]. Alterations in the composition of the intestinal microbiota are associated with changes in the inflammatory, neuroendocrine and behavioral responses of the host [8]. Early life adversity such as MS disturbs development and composition of the intestinal microbiota [9]. Alterations in the composition of the microbiota, along with increasing gut permeability following stress, could lead to the over-production of pro-inflammatory cytokines in the bowel. It is coupled with the fact that there are noticable interactions between stress, the immune system and the gut microbiota [10].

It has been suggested that oxidative and nitrosative stress (O&NS), as well as immune-inflammatory responses, have an imperative role in the pathophysiology of psychiatric disorders [11]. Moreover, chronic exposure to glucocorticosteroids could initiate inflammatory immune responses in the brain and arise depressive-like behaviors [12].

Considering 1) the relationship between stress and immune-inflammatory responses following early life tensions, 2) Alterations in microbiota following chronic stress, 3) over-activation of the HPA axis in stressful condition, 4) potential role of neuroinflammation in the pathophysiology of psychiatric disorders; in the present study, we aimed to evaluate the role of corticosterone on microbial composition, neuroinflammation and behavioral changes following maternal separation stress. In addition, we intended to examine whether adrenalectomy can modulate the negative effects of MS.

2. Materials and methods

2.1. Animals and housing conditions

Forty-five pregnant NMRI mice on gestation day of 1 were bought from Pasteur Institute of Iran. Animals were kept under standard laboratory environment of 12-h light/dark cycle, temperature 22 ± 1 °C and free access to food and water. The day of birth was considered as postnatal day (PND) 0. Pups at PND 2 were subjected to maternal separation (MS) paradigm. In this model, offsprings were daily separated from their mothers during PND 2–14 for 3 h (09:00–12:00 a.m.) and then returned to their mothers' cages [13]. At the end of PND 14, pups were returned to their mothers' cages and stayed untouched until PND 21. On PND 21, male mice were weaned and kept in separate groups (4 mice per cage) until experiment day at PND 50. Another group of control animals was left undisturbed until PND 50.

All procedures in this study were done in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication # 80-23) and institutional guidelines for animal care and use (Shahrekord University of Medical Sciences). All tests were performed between 08:00 a.m. to 02:00 p.m. Each experimental group contained 6 to 8 animals for behavioral assessments and 3–4 mice for molecular tests. We tried to minimize the use of animals.

2.2. Study design

The study was performed in two different steps. For the first step, in order to evaluate the impact of early life stress on mice behavior we carried out valid behavioral tests related to depressive- and anxiety-like behaviors including open filed test (OET), forced swimming test (FST), splash test, and elevated plus maze (EPM). At the end of this step, the animals were sacrificed, Colon and blood samples were taken and hippocampi were dissected out on an ice-cold surface and directly snapped freeze in liquid nitrogen and kept in −80 °C until the start of molecular assays. Furthermore, the expression of inflammatory genes, as well as histopathological changes in the colon, was assessed. In addition, the composition of the intestinal microbiota from stool samples was evaluated in this step.

On the second part, for evaluation of the role of the HPA axis and corticosterone on the enteric microbial compositions and behavioral alterations, mice were subjected to adrenalectomy (PND 40) and after 10 days the above-mentioned evaluations (step 1) were performed as well.

All behavioral experiments were performed in adult male mice (PND 50–52) and each mouse was used only for one test.

2.3. Behavioral experiments

2.3.1. Forced swimming test (FST)

The FST was performed to assess despair behavior. In this trial, increased immobility time reflected despair behavior, as a principal symptom of depression, in mice [14]. In this regard, animals were individually located in a cylinder (diameter: 10 cm, height: 25 cm) containing 19 cm water (23 ± 1 °C). Mice were enforced for 6 min swimming and an investigator blinded to the treatment or environmental conditions documented the immobility time during the last 4 min of the test. Immobility time was described as the duration of time that the mouse remained immobile in the water and made only the essential movements to maintain its head above.

2.3.2. Open-field test (OFT)

The OFT was done to investigate the effects of MS and adrenalectomy on motor function [15]. The OFT apparatus was made of dimly illuminated white opaque Plexiglas (50 cm × 50 cm × 30 cm). Each animal was mildly placed on the central area of the device (30 cm × 30 cm) and using a camera, locomotion was recorded for 5 min and evaluated by Ethovision software version 8 (Noldus, Netherlands). In the OFT the distance moved (horizontal activity) and the number of rearings (vertical activity) was measured. Ethanol 70% was used to clean the apparatus after testing of each mouse.

2.3.3. Splash test

The splash test was carried out to assess self-care and motivational difficulties. In this test, grooming activity time in response to a spray of sucrose solution was considered as an indirect criterion of palatable solution intake. A 10% sucrose solution was sprayed on the dorsal coat of mice and mice were videotaped for 5 min. Nose/face grooming, head washing, and body grooming were considered as grooming activity behaviors [16].

2.3.4. Elevated plus maze (EPM)

The elevated plus maze (EPM) is an approved trial for evaluation of anxiety in rodents [17]. The apparatus was made of black opaque Plexiglas and involved of two open (30 × 5 cm) and closed (30 × 5 × 15 cm) arms, which were joined by a platform area (5 × 5 cm). Testing room was faintly illuminated and mice were separately sited in the center of the EPM facing to closed arm and mice were videotaped for a 5 min period. The apparatus was cleaned with 70% ethanol after testing each mouse. The total time spent in the open arms as well as numbers of entries into the open arms were recorded and reported as percentages.

2.4. Real-time RT-PCR analysis for assessment of inflammatory genes

TRIzol reagent (Invitrogen) was used to extract total RNA from hippocampi and bowel tissue. After the reverse transcription of 1 μg of RNA using PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan), qRT-PCR was done to determine alterations in the mRNA levels of favorite genes. qRT-PCR was done on a light cycler device (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex Taq technology (Takara Bio). Thermal cycling conditions were; including an initial activation step for 30 s at 95 °C afterward 45 cycles as well as a denaturation step for 5 s at 95 °C and a combined annealing/extension step for 20 s at 60 °C. Melting curve analysis was done to confirm whether all primers generated a single PCR product. The genes and their primers are listed in Table 1. Histone H2A variant, H2afz, was

Table 1
Primer sequences for Real time PCR.

Primer	Forward sequence	Reverse sequence
H2afz	TCATCGACACCTGAAATCTAGGA	AGGGGTGATACGCTTTACCTTTA
Tnf-α	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
Il-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
Tlr4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA
Myd88	ATCGCTGTTCTTGAACCCTCG	CTCACGGTCTAACAAGGCCAG
Nlrp3	ATCAACAGGCGAGACCTCTG	GTCCTCCTGGCATACCATAGA
Inos	TTTGACCAGAGGACCCAGAG	AAGACCAGAGGCAGCACATC
Hmgb1	GCTGACAAGGCTCGTTATGAA	GCTGACAAGGCTCGTTATGAA
Lactobacillus	CTCGTTGCGGGACTTAA	GCAGCAGTAGGGAATCTTC
bifidobacterium	CTCCTGGAAACGGGTGG	GGTGTTCTTCCCGATATCTACA
Clostridium coccoides	AAATGACGGTACCTGACTAA	CTTTGAGTTTCATTCTTGCGAA
Bacteroides fragilis	ATAGCCTTTCGAAA	ATTTTAACGTCAACTATGACC
Prevotella	CACRGTAAACGATGGATGCC	GGTCGGGTTGCAGACC
Clostridium leptum	GCACAAGCAGTGGAGT	CTTCCTCCGTTTTGTCAA

used as normalizer gene and variations in expression of each mRNA in comparison with H2afz was measured based on $2-\Delta\Delta Ct$ relative expression formula, as described previously [18].

2.5. Bacterial DNA extraction from stool

Mice were euthanized, colon opened longitudinally and stool samples were collected. Total bacterial DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit (Qiagene, Venlo, Netherlands) according to the manufacturer's instructions. After determination of DNA concentration by NanoDrop, DNA samples were diluted and using Real-time RT-PCR method composition of *Lactobacillus*, *Clostridium coccoides*, *Bifidobacterium*, *Bacteroides fragilis*, *Prevotella* and *Clostridium leptum* species were determined.

2.6. Corticosterone assay

Blood samples were collected and plasma was separated using cold centrifugation. Plasma corticosterone level was assayed by corticosterone ELISA kit (Enzo Life Sciences; PA, USA) according to the manufacturer instructions.

2.7. Histopathological evaluation

For histopathologic assessment, the colon was cut into bits and fixed in 10% formalin. Formalin-fixed samples were paraffin-embedded and cut into 5-μm sections. Nine sections obtained from each colon and were deparaffinized using xylene and discolored with hematoxylin and eosin (H&E). According to the previous criteria [2], histological analysis was done by a pathologist blinded to the study. Each score characterized the mean of nine sections of each colon. Histopathology was scored as follows: Epithelium (E): 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas. Infiltration (I): 0, no infiltrate; 1, infiltrate around crypt basis; 2, infiltrate reaching to L. muscularis mucosae; 3, extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant edema; 4, infiltration of the L. submucosa. The total histological score represents the sum of the epithelium and infiltration score (total score = E + I).

2.8. Adrenalectomy

Mice were subjected to anesthesia with sodium thiopental (100 mg/kg, i.p.). The skin of the back was shaved and disinfected using betadine. Through a dorsal lateral incision, adrenals were subsequently removed bilaterally from the surrounding tissues. Subsequently, the cuts in the muscle walls and flank incision were closed with silk sutures. Adrenalectomized mice were placed on a warm incubation chamber for recovery. After surgery, mice were maintained on 0.9% NaCl as drinking water to maintain mineral balance and were allowed 10 days for surgical recovery prior to experiments [19,20].

2.9. Statistics

Comparison between the groups was analyzed using two-way ANOVA followed by Bonferroni post hoc test in the GraphPad Prism software (version 7). P-value < 0.05 was considered statistically significant. The sample size was calculated by power calculations using G power software (ver.3.1.7, Franz Faul, Universitat Kiel, Germany). We set α error at 0.05 and power (1-β) at 0.8 and the required total sample size per group was calculated as 6–8 in behavioral tests and 3–4 for molecular experiments.

3. Results

3.1. Adrenalectomy could modulate depressive -like behaviors in adult male mice

Two-way ANOVA analysis showed that there are significant differences between experimental groups. Bonferroni post-test showed that the duration of immobility time in the FST (Fig. 1A) significantly increased in MS mice in comparison with the control group ($P < 0.01$). Moreover, following adrenalectomy duration of immobility time significantly decreased in MS mice ($P < 0.05$). In the splash test (Fig. 1B), results showed that MS provoked a significant decrease in grooming activity time in comparison with control mice ($P < 0.01$). Post-test analysis revealed that grooming activity time in adrenalectomized MS mice significantly increased when compared with MS counterpart ($P < 0.05$). In the OFT, findings of post analysis determined that the horizontal activity ($P < 0.01$, Fig. 1C), and the number of rearings (vertical activity) ($P < 0.05$, Fig. 1D) significantly increased in MS mice when compared with the control mice. Our findings showed that unlike vertical activity (Fig. 1D), horizontal activity considerable decreased in MS mice underwent adrenalectomy rather than the MS counterpart group ($P < 0.05$, Fig. 1C). The amount of time and the number of entries to the open arm of EPM device were much lower in maternally separated mice compared to the control counterpart ($P < 0.001$, Fig. 1E and F). Results obtained from Bonferroni analysis determined that both open arm time and open arm entries significantly increased in MS mice were underwent adrenalectomy surgery ($P < 0.05$, for both). In addition, in aspect of behavioral assessments, we found that there are significant differences between adrenalectomized-MS mice with adrenalectomized-control mice in cases of immobility time in the FST ($P < 0.05$), vertical activity in the OFT ($P < 0.05$) and open arm entries in the EPM ($P < 0.05$).

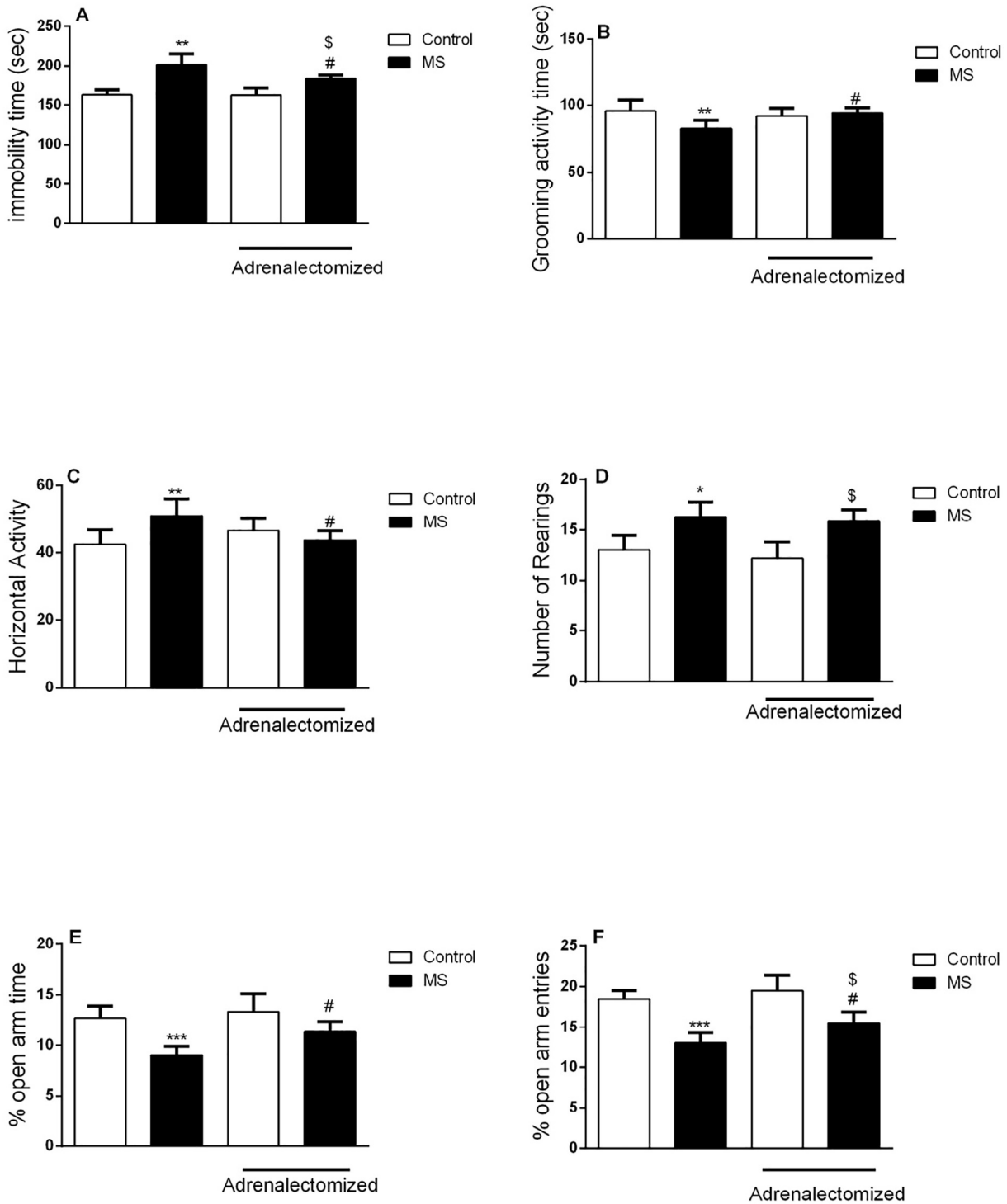

Fig. 1. Effects of MS and adrenalectomy on anxiety- and depressive-like behaviors in male mice: FST (A), splash test (B), OFT (C and D) and EPM (E and F). Values are shown as the mean ± S.E.M for 6 to 8 animals and were analyzed using two-way ANOVA followed by Bonferroni post hoc test. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the control group, $^{\#}$P < 0.05 indicates comparison with the non-adrenalectomized MS group, and $^{\$}$P < 0.05 compared with the adrenalectomized-control mice.

3.2. Adrenalectomy could modulate the expression of genes are relevant to immune-inflammatory responses in the hippocampus

The expression of genes related to neuro-immune responses in the hippocampus is shown in Fig. 2. Two-way ANOVA analysis showed that there were significant differences between experimental groups. Bonferroni analysis demonstrated that expression of the *Il-1ß* (P < 0.001), *Myd88* (P < 0.01), *Tlr4* (P < 0.01), *Nlrp3* (P < 0.01) and *Hmgb 1* (P < 0.001) but not *Tnf-α* and *iNOS* significantly increased in the hippocampus of MS mice in comparison with control mice. Results showed that MS mice adrenalectomy significantly mitigated expression of *Il-1ß*, *Myd88*, *Tlr4*, *Nlrp3* and *Hmgb 1* in the hippocampus in comparison with the intact counterparts (P < 0.05 for all). However, adrenalectomy did not lead to significant changes in the expression of inflammatory genes in the control group. Furthermore, findings showed that there are remarkable differences between control and MS mice

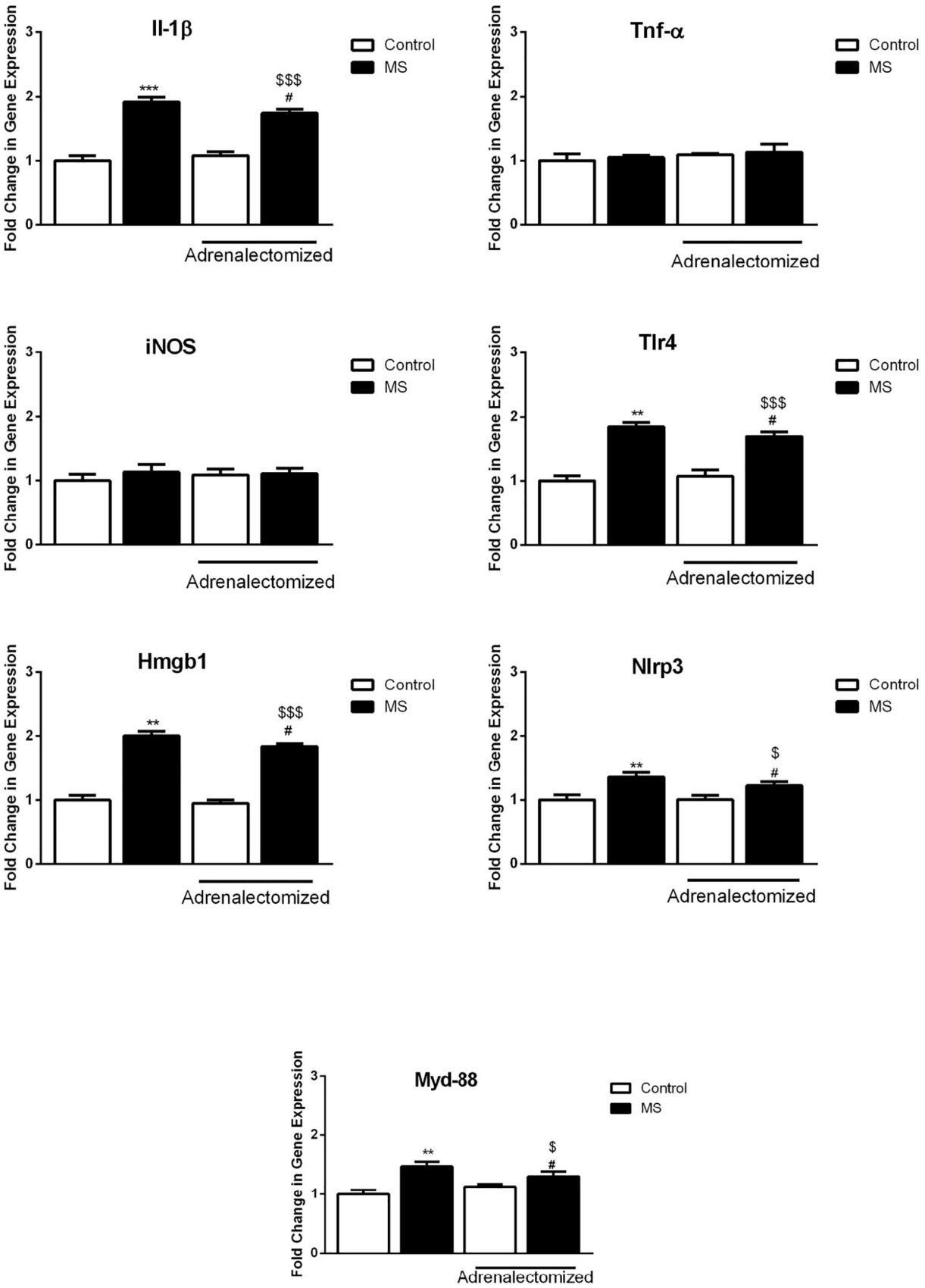

Fig. 2. Effects of MS and adrenalectomy on the gene expression of immune-inflammatory markers (*Il-1β*, *Tnf-α*, *iNOS*, *Tlr4*, *Hmgb1*, *Nlrp-3*, and *Myd88*) in the hippocampus of the mice. Data are expressed as the mean ± S.E.M and were analyzed by two-way ANOVA. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the control mice and $^{\#}P < 0.05$ compared with non-adrenalectomized MS mice, $^{\$}P < 0.05$ and $^{\$\$\$}P < 0.001$ compared with adrenalectomized-control mice.

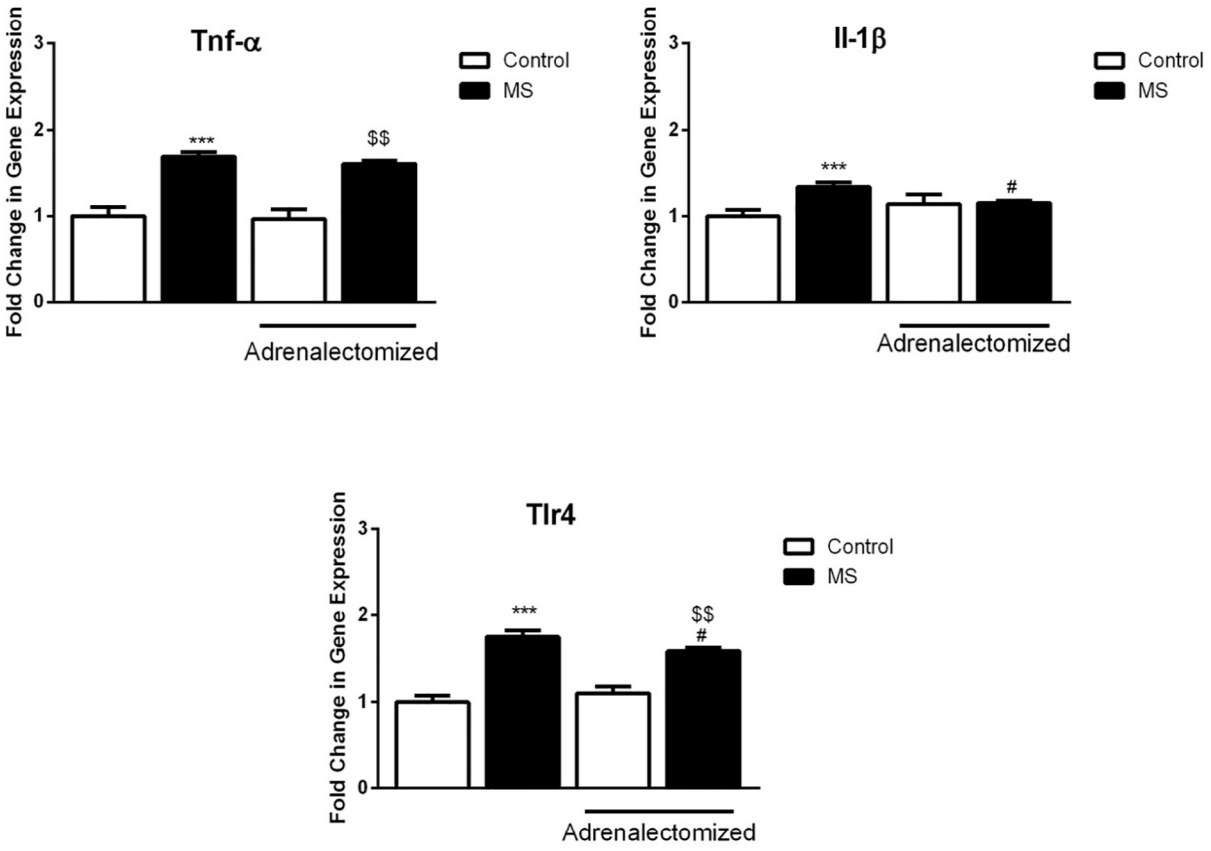

Fig. 3. Effects of MS and adrenalectomy on the gene expression of inflammatory cytokines (*Il-1β*, *Tnf-α,* and *Tlr4*) in the colon tissue of the mice. Data are expressed as the mean ± S.E.M and were analyzed by two-way ANOVA. ***P < 0.001 indicates comparison with the control mice and $^{\#}$P < 0.05 indicates comparison with non-adrenalectomized MS mice, $^{\$\$}$P < 0.01 compared with adrenalectomized-control mice.

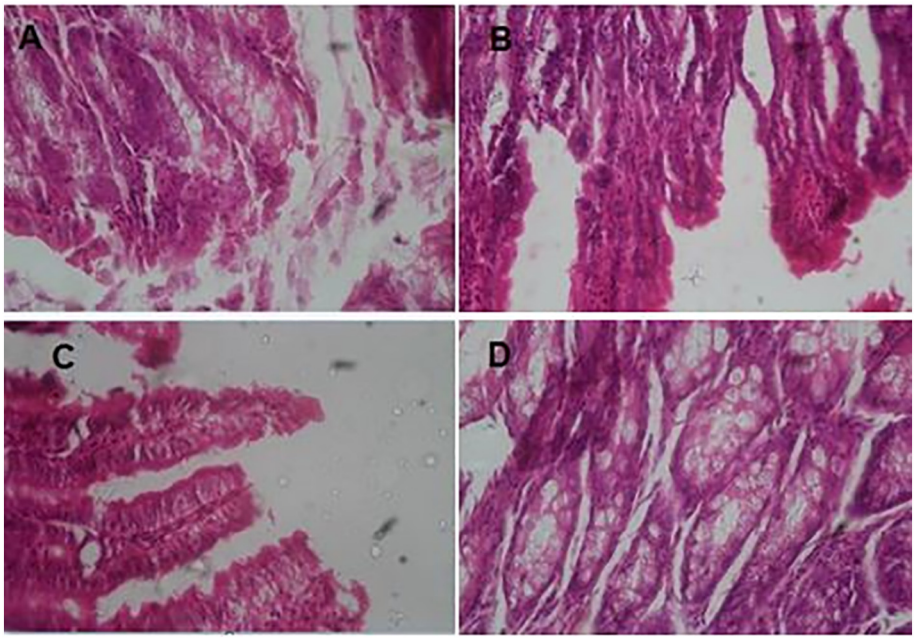

Fig. 4. Representative features of histopathologic evaluations provided by H & E-stained colon sections (×100). A: The control group; B: the MS group; C: the adrenalectomized control group; D: the adrenalectomized MS group. The normal mucus layer and crypts without leucocyte infiltration observed in the control group while the mucosal layer with leucocyte infiltration observed in the MS group.

underwent adrenalectomy (P < 0.05 and P < 0.001).

3.3. Adrenalectomy could modulate the expression of genes are related to inflammation in the bowel tissue

As shown in Fig. 3, expression of *Tlr4* (P < 0.001), *Tnf-α* (P < 0.001) and *Il-1β* (P < 0.01) in the bowel tissue significantly increased in the MS group compared to the control group. Additionally, adrenalectomy significantly declined expression of *Tlr4* (P < 0.01) and *Il-1β* (P < 0.05) (but not *Tnf-α*) in colon tissue of the MS mice. Furthermore, we did not detect significant changes in the control mice undergoing adrenalectomy, compared to the intact counterpart. Also,

findings showed that there are significant differences between control and MS mice underwent adrenalectomy ($P < 0.01$).

3.4. Adrenalectomy reduced the level of corticosterone in the plasma samples

Results showed that the concentration of corticosterone in the MS group (367.8 ± 6.46) was significantly greater than the control group (130.3 ± 4.2) ($P < 0.001$). In addition, the level of corticosterone in adrenalectomized MS mice (283.5 ± 4.7) was significantly decreased when compared with MS counterpart ($P < 0.01$). However, adrenalectomy did not cause significant alterations in the aspect of corticosterone concentration in control animals (114.6 ± 3.8). Also, we found that there is significant different between adrenalectomized-MS mice with adrenalectomized-control mice ($P < 0.001$).

3.5. Adrenalectomy could not modulate MS- induced histopathologic changes in the colon

As shown in Fig. 4, epithelial damage and inflammatory cell infiltration were detected in the MS group. Neutrophilic permeation limited to the mucosa, goblet cell, and crypt loss was obvious, indicating a colonic injury. Moreover, adrenalectomy failed to modulate the damaging effects of MS on colon tissue. Overall, the histopathological scores as (median (min-max)) were significantly higher in the MS group (3 [2–4]) in comparison with the control group (1 (0–2)) ($P < 0.05$). Findings showed that adrenalectomy failed to change the histopathologic scores either in the MS group (3 [2,3]) or the control group (1 [1,2]) when compared with their counterparts.

3.6. Adrenalectomy could restore composition of the gut microbiota in MS mice

Our findings showed that following MS paradigm populations of *Bifidobacterium bifidum*, *Lactobacillus*, *Clostridium leptum* and *Clostridium coccoides* (but not *Prevotella* and *Bacteroides fragilis*) increased in compared to control mice ($P < 0.05$, Table 2). Comparison between MS mice underwent adrenalectomy with their MS counterparts demonstrated that adrenalectomy reinstates aforementioned bacteria populations in the gut ($P < 0.05$). Also our results showed that there are significant differences between control and MS mice underwent adrenalectomy for *Bifidobacterium* ($P < 0.01$) and *Clostridium leptum* ($P < 0.05$).

4. Discussion

Findings of the present study showed that maternal separation (MS) stress provoked anxiogenic- and depressive-like effects in adult male mice revealed by behavioral tests including FST, OFT, EPM and splash test. We observed that the expression of inflammatory genes related to neuro-immune response increased in the hippocampus of MS mice. Evaluation of the bowel (colon) tissue determined that MS led to the histopathologic changes in the colon and also increased the expression of inflammatory genes. Our results indicated that MS changed the composition of microbiota in the bowel. Moreover, we found that corticosterone mediated the negative effects of MS. In this regards, our data demonstrated that adrenalectomy could modulate the harmful effects of MS on behavior, colon tissue and also the composition of bowel microbiota.

Social ties, principally mother-infant synchrony during the infantile period, have a critical role in the development of the brain and behavior in the adulthood [1]. Previous researches have revealed that the quality of early life is intensely connected with psychological well-being in later life. In this regards, it has been determined that experiencing stress in the neonatal stage of life is associated with adulthood psycho-affective disorders including depression and anxiety [21]. Experiencing early psychological tensions, like MS stress, modifies the neurotransmission systems, cause structural and functional alterations in the brain which subsequently lead to the psychiatric disorders [2]. Ample studies on animals subjected to different types of stress have revealed that stress led to deficits in the open field activity [22,23]. Indeed, we performed the OFT to confirm that locomotor activity following different conditions does not affected results of the FST [24]. We did the FST as a valid behavioral test for assessment of passive behavior in rodents. In this test, increase in immobility time translate the behavioral despair [25]. The splash test is a valid test for assessing the self-care and motivational difficulties in rodents. In this paradigm, decrease in grooming activity time indicated self-care deficit [26]. The EPM is an approved test performed to evaluate the anxiety-like behavior in rodents in which decrease in frequency and spent time on open arms reflects the anxious state [27]. In consistent with previous studies, our results showed that MS stress led to depressive- and anxiety-like behaviors in adult male mice [28,29]. MS increased the duration of immobility time in the FST, increased horizontal and vertical activities in the OFT, decreased grooming activity time in the splash and also decreased the time and number of entries to the open arms in the EPM test.

It has been obvious that glucocorticoids, as main products of the HPA axis, stimulate adaptation and retrieval from stress and restore homeostasis following exposure to stresses [30]. However, endurable stimulation of the HPA axis and increase in glucocorticoid levels disturb physiological functions which subsequently appear adverse health consequences [31,32]. Overactivation of the HPA axis and increase corticosterone levels are attributed to the Suffering adversity in early life [3]. In this regard ample evidence determined that early-life stresses negatively affected the later life welfare [21,33]. In line with the aforementioned studies, our results demonstrated that the level of corticosterone significantly increased in the serum samples of MS mice which are in accordance with the behavioral changes following MS stress.

The gut contains a diverse microbial biota, which are mainly anaerobes bacteria, in addition to fungi and viruses [34]. It is becoming more evidence to indicate that commensal microbiota in the gut plays a pivotal role in primary programming and adulthood responsivity of the

Table 2
Mean CT ± SD for RT-PCR results of microbiota populations.

	Control	MS	Control adrenalectomized	MS adrenalectomized
Lactobacillus	27.68 ± 0.15	29.04 ± 0.11*	24.96 ± 0.08	26.41 ± 0.13$^{\#}$
Bifidobacterium	23.1 ± 0.14	29.89 ± 0.11*	16.96 ± 0.09	24 ± 0.1$^{\#, \$\$}$
Clostridium coccoides	32.12 ± 0.14	36 ± 0.05*	31.8 ± 0.16	28.16 ± 0.13$^{\#}$
Bacteroides fragilis	27.79 ± 0.14	28.09 ± 0.11	26.74 ± 0.07	28.3 ± 0.12
Prevotella	19.11 ± 0.12	19.05 ± 0.08	19.46 ± 0.14	18.73 ± 0.07
Clostridium leptum	33.17 ± 0.1	39.34 ± 0.18*	32.18 ± 0.13	28.25 ± 0.08$^{\#, \$}$

Effects of MS and adrenalectomy on the composition of gut microbiota (*Bifidobacterium bifidum*, *Lactobacillus*, *Clostridium leptum*, *Clostridium coccoides*, *Prevotella* and *Bacteroides fragilis*). Data are expressed as the mean ± SD and were analyzed by two-way ANOVA. *$P < 0.05$ compared to the control mice and $^{\#}P < 0.05$ compared to non-adrenalectomized MS mice, $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ compared with adrenalectomized-control mice.

stress system [35,36]. Microflora has a central role in the regulation of emotions and behavior [7]. Alterations in the composition of the intestinal microbiota are associated with changes in the inflammatory, neuroendocrine and behavioral responsiveness of the host [8]. In respect to the bidirectional connections between the central nervous system and the enteric nervous system with the gastrointestinal tract, investigations suggest the existence of a critical role for the gut microbiota in the brain-gut axis [37]. In this regards, evidence showed that the intestinal microbiota influences the brain and behavior [38]. Early life adversity such as MS disturbs development and composition of the intestinal microbiota and augments production of inflammatory cytokines [9,33]. Results of the present study showed that composition of the gut microbiota is changed as a result of MS. We found that MS significantly increased the population of *Bifidobacterium bifidum*, *Lactobacillus*, *Clostridium leptum*, and *Clostridium coccoides*. Moreover, adrenalectomy could modulate the effects of MS on gut microbiota composition. According to data obtained from adrenalectomized mice, we could intensively conclude in this part that overactivation of the HPA axis and overproduction of corticosterone is responsible for changes in the composition of microbiota.

It has been determined that activation of the HPA axis increased the gut permeability [39]. Considering that MS stress lead to the dysfunction of the gut-brain axis, MS can be considered as a valid model to study the underlying mechanisms linked the behavioral disorders with gut disorders like irritable bowel syndrome (IBS) [33,40]. There is a notable cross-talk among stress, immune system and the gut microbiota [10,41]. Mast cells, through secretion of interleukins, play an imperative role in mediating the impact of stress on the gut [42,43]. It has been demonstrated that stress increase the expression of proinflammatory cytokines in the gut mucosa [10,38]. Commensal bacteria are responsible for immunologic accommodation to microflora in the healthy gut. These bacteria are vital for functional integrity of the mucosa and also protection against pathogens [44]. On the other hand, under stressful conditions and change in the composition of commensal bacteria, enteric bacteria are presented to the mucosal immune cells and initiate an inflammatory response in the gut [38]. In agreement with the aforementioned studies, our findings showed that expression of genes related to inflammation including *Il-1β*, *Tnf-α*, and *Tlr-4* in the colon tissue significantly increased in following MS stress. We found that adrenalectomy mitigated the expression of inflammation-relevant genes in the colon tissue. The histopathologic evaluation demonstrated that MS paradigm led to the damage and infiltration of immune cells to the colon tissue. Furthermore, we showed that adrenalectomy (after 10 days) decreased the harmful histopathological effects of MS on the colon tissue.

It has been well-determined that stress induces immunomodulatory functions through modifications in the HPA axis and the sympathetic nervous system [45]. Developing line of evidence suggested that oxidative and nitrosative stress (O&NS) and immune-inflammatory responses are the main cause of the pathophysiology of psychiatric disorders [2]. oxidative stress and proinflammatory cytokines are exclusively involved in the pathophysiology of gastrointestinal diseases such as IBD [2]. Peripheral inflammation in the brain is able to initiate the development of psychiatry disorders via activation of immune-inflammatory responses [46]. Furthermore, overexpression of innate immune genes such as IL-1β, IL-6, TNF, Toll-like receptor (TLR4), has been detected in brain samples collected from suicide victims of depression [45]. TLRs as the main components of innate immunity recognizes the microbial-associated molecular patterns) MAMPs) such as lipopolysaccharide (LPS) and damage-associated molecular patterns (DAMPs) such as Hmgb1. It has been well-determined that activation of TLRs initiates production of pro-inflammatory cytokines in the brain. In this concept, previous studies have demonstrated that stress activates pattern recognition receptors (PRPs) like TLRs and NLRP3 inflammasome [47]. Blockade of NLRP3 converses stress-induced increases in inflammatory cytokines in the peripheral blood and brain as well as reverses depressive-like behaviors [48]. There is evidence suggested that inflammasomes mediated the role of the gut microbiome in mood regulation [49]. As a consequence of stress, commensal bacteria, MAMPs and DAMPs can activate TLRs and NLRP3 in the peripheral monocytes of the gut. Pro-inflammatory and inflammatory cytokines are produced in the peripheral monocytes via cellular, humoral and neural routes reach to the brain and activate microglia and macrophages resulting in neuro-immune response in the brain [50]. Recently, it has been documented that neuroinflammation and immune-inflammatory response in the brain, hippocampus in particular, mediated the negative effects of stress on animal's behaviors [2,51]. In line with above-mentioned studies our data determined that MS stress as a valid model of early life stress increased the expression of genes related to immune-inflammatory response including *Il-1ß*, *Myd88*, *Tlr4*, *Nlrp3* and *Hmgb 1* in the hippocampus. These results indicated that depressive- and anxiogenic-like behaviors following MS paradigm are associated with neuroinflammatory response in the hippocampus. Furthermore, we demonstrated that corticosterone, at least in part, could mediate neuroinflammation and subsequently behavioral changes in MS mice. In this regards, we showed that adrenalectomy could decrease neuroimmune response in the hippocampus and restore microbiota composition in the gut and probably by these mechanisms, may be at part, could mitigate depressive- and anxiogenic-like behaviors of MS mice. The important point of this study is that, as previous studies have determined, early-life stress, such as MS, leads to persistent and life-long overactivation in the offspring's HPA stress axis [4,5]. Our findings showed that adrenalectomy, would modulate this persistent hyperactive response and by this way, probably, modulate negative effects of MS. It is important to note that adrenalectomy, partially at least, could mitigate the negative effects of MS stress on behavioral disorders, (neuro) inflammatory responses as well as change in microbiota composition. Findings of the present study determined that following adrenalectomy adverse effects of chronic early life stress could be diminished, in part, in the MS mice. However, we observed significant differences between the adrenalectomized-MS mice with the adrenalectomized-control mice which warranted further studies to clarify the reason of these differences. Also, further studies should be done in the future to exactly determine the role of corticostrone in mediating the negative effects of MS.

5. Conclusion

In conclusion, results of the present study demonstrated that 1) MS provoked depressive- and anxiety-like behaviors in adult male mice 2) MS modified gut microbiota composition 3) MS activated neuro-immune response in the hippocampus 4) MS led to the damage in colon tissue 5) MS led to overproduction of corticosterone 6) adrenalectomy inverted, at least in part, negative effects of MS on the behavior and also mitigated neuroinflammatory response in the hippocampus 7) adrenalectomy reset, may be partially, the microbiota composition in the gut. Overall our findings showed that behavioral changes, neuroinflammation and changes in microbiota composition following MS stress, at least partially, are mediated by corticosterone.

Conflict of interest

The authors declare that there is no conflict of interest.

Compliance with ethical standards

All applicable international and institutional guidelines for the care and use of animals were followed.

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